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Selection and Identification of Phosphate-Potassium Solubilizing Bacteria from the Area Around the Limestone Mining in Cirebon Quarry

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ABSTRACT

Phosphorus (P) and potassium (K) are essential for the growth and development of plants. Most of phosphate and potassium are bound in the form of rocks P or K-minerals which not be directly absorbed by plants. The utilization of microbes including Phosphate Solubilizing Bacteria (PSB) and Potassium Solubilizing Bacteria (KSB) is deemed as an alternative to address the issue of P and K availability. The objective of the research was to select and identify the phosphate-potassium solubilizing bacteria and measure the quantitative estimation of solubilized P and K. Phosphate-potassium solubilizing bacteria were collected and regrown using dot method on Pikovskaya solid medium to solubilize P and on Aleksandrov solid medium to solubilize K, incubated for 7 days at room temperature. The QC3.a.1 and QC3.d.5 showed the highest solubilization index on Pikovskaya medium containing $Ca_3(PO_4)_2$, while QC3.a.2 produced the highest amount of soluble phosphate. The QC3.a.2 showed the best of potassium solubilizing activity based on clear zone formation, while QC3.a.1 had the best ability in feldspar hydrolyzation. The three strains were Gram-negative bacteria. Phylogenetic analysis based on 16S rRNA gene sequence showed that isolate QC3.a.1 was closely related to *Burkholderia*, isolate QC3.a.2 was closely related to *Serratia* and isolate QC3.d.5 was closely related to *Pseudomonas putida*.

Key words: Phosphate-potassium solubilizing bacteria, solubilization index, phosphate-potassium solubilization, 16S rRNA gene, Cirebon Quarry

INTRODUCTION

Phosphorus (P) and Potassium (K) are important macronutrients that necessary for the growth and development of plants. The needs of P and K in plants are usually obtained through fertilizer application. However, such effort became inefficient because P is bound to Fe-P or Al-P in acid soil or Ca-P in alkaline soil (Cunningham and Kuiack 1992) and K is easily leached by water in the soil, so that P and K are unavailable for plants. The inefficient P and K fertilizer can be solved by using Phosphate Solubilizing Bacteria (PSB) and Potassium Solubilizing Bacteria (KSB), as biological fertilizer.

Most of phosphate and potassium in the soil are bound in the form of rocks P, K-minerals and other deposits (Goldstein, 1994), which can not be absorbed by plants. Phosphate and potassium are only available for plants, when present in the soluble form (Bertsch and Thomas, 1985; Kpomblekou-A and Tabatabai, 1994). Phosphate plays an important role for the process of photosynthesis and nutrient uptake (Illmer and Schinner, 1992), while the potassium plays an

important role for the growth and reproduction of plants (Badr, 2006) and the turgidity of guard cells (Shehata and El-Khawas, 2003).

Phosphate Solubilizing Bacteria (PSB) is capable to convert insoluble phosphate in to soluble forms through the production of organic acids, chelates formation, exchange reaction (Chen *et al.*, 2006). Among bacteria strains capable to solubilize P are *Pseudomonas*, *Bacillus*, *Enterobacter*, *Azotobacter*, *Agrobacterium*, *Achromobacter*, *Rhizobium*, *Burkholderia*, *Flavobacterium* and *Micrococcus* (Son *et al.*, 2006).

Potassium Solubilizing Bacteria (KSB) can dissolve K-minerals such as mica, illite and orthoclase in the soil through the production and excretion of organic acids or production of capsular polysaccharide (Friedrich *et al.*, 1991; Sheng and He, 2006). Several strains of potassium solubilizing bacteria, such as *Pseudomonas*, *Burkholderia*, *Acidothiobacillus ferroxidans*, *Bacillus mucilogenosus*, *B. edaphicus*, *B. circulans* and *Paenibacillus* sp. (Lian *et al.*, 2002; Sheng, 2005; Li *et al.*, 2006; Liu *et al.*, 2012) can be used as biofertilizer.

Indonesia is one of the countries having many sites of open-pit mining system. One of the Indonesian open-pit mining is limestone mining (quarry) Palimanan, Cirebon. Open-pit mining can changes the physical, chemical and biological aspects of soil. Obtaining the desired target in revegetation of ex-mining areas are usually difficult because of the reduction of microbes potential or the low growth rate of plants which correlated to the lack of nutrients availability in the soil. The PSB and KSB can be potentially useful, as an alternative solution in addressing the problem of P and K availability in ex-limestone mining reclamation area and can be developed as biological fertilizers. This bacteria have beneficial value for soil fertility and plants cultivated on ex-mining land. This research was focused on selection and identification of phosphate-potassium solubilizing bacteria and quantitative estimation of P and K solubilized by bacteria isolated from the area around the limestone mining of Cirebon Quarry. This research was conducted in relation to the effort of limestone mining reclamation.

MATERIALS AND METHODS

Testing bacteria ability to solubilize P and K: Phosphate-potassium solubilizing bacteria were collected from Mubarik *et al.* (2014) and regrown using dot method on Pikovskaya solid medium (Nautiyal, 1999) to solubilize P and on Aleksandrov solid medium (Prajapati and Modi, 2012) to solubilize K, incubated for 7 days at room temperature. After incubation, the clear zone (halozone) formed around colonies were measured. Start from this sentence, corrected with: Solubility Index (SI) for P and K was calculated by subtracting the value of the diameter of halozone toward diameter of bacterial colony and then the result divided to the diameter of the colony:

$$SI = \frac{\text{Halozone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

Quantitative estimation of soluble P and K: Quantitative estimation of P solubilized by the selected isolates was conducted by following Lynn *et al.* (2013). Isolates selected (QC3.a.1, QC3.a.2 and QC3.d.5) were cultivated in 100 mL Pikovskaya liquid medium and incubated in shaking incubator for 7 days at 37°C. Every 24 h, 1.5 mL bacteria culture was centrifuged at 10,600 g for 10 min. One milliliter of supernantant was reacted with the color-forming reagent (2.5 mL 2.5% sodium molybdate and 1 mL 0.3% hydrazine sulfate). After blue color formed, phosphate solubilizing activity was then measured by visible spectrophotometer (Spektro Genesys 20) at 830 nm wavelength.

Quantitative estimation of solubilized K was conducted by following, Parmar and Sindhu (2013). The three selected isolate were cultivated in 50 mL Aleksandrov liquid medium and incubated at 28°C in shaking incubator for 25 days. Every 5 days, 3 mL bacteria culture was centrifuged at 10,600 g for 10 min. The amount of soluble potassium in the supernatant was measured by atomic absorption spectrometer (AA-7000 AAS with flame air-C_2H_2) at 766.5 nm wavelength.

Bacterial morphology and physiology: Morphological identification of selected isolate was conducted using streaked plate method on Nutrient Agar (NA) medium and incubated for 48 h at room temperature. After incubation, colony morphology such as shape, edge, elevation and the colony color was observed. Gram staining then was conducted to define bacteria cells type and form (Hadioetomo, 1993). Physiological identification was conducted using Analytical Profile Index (API) 20 NE/E biochemical test kit method (bioMerieux, Inc, Durham, USA). The results then were analyzed using API 20 NE/E Table and API 20 NE/E program (software) to determine the species of isolates tested.

Isolation of bacterial DNA genome: Single bacteria colony from selected isolates on NA medium was grown in 50 mL Luria Broth (LB) medium and incubated in shaking incubator at 150 rpm and 37°C for 24 h. The bacteria cells were harvested using centrifugation at 10,600 g for 10 min. Bacteria DNA genome was extracted using protocol of PrestoTM gDNA Bacteria Mini Kit (Geneaid).

Amplification of 16S rRNA gene: Polymerase chain reaction mix of 50 µL was composed by 10 pmol 63f and 10 pmol 1387r primers of 3 µL each (Marchesi *et al.*, 1998), 25 µL GoTaq Green Master Mix 2x (Promega, Madison, W1, USA), 12 µL ddH_2O and 7 µL DNA template. The PCR was performed in 30 cycles with: predenaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 55°C for 1 min, elongation at 72°C for 1.5 min and post elongation at 72°C for 10 min. After EtBr staining was conducted, the PCR product was then visualized under UV light using 1% gel agarose electrophoresis of 75 volt for 46 min.

Sequences DNA and filogenetic analysis: The DNA sequences were then compared with the sequences available in public databases of NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST) using the BLAST-N program. Phylogenetic analysis was performed using Mega 5.05 program and Neighbour Joining (NJ) method with bootstrap 1000x.

RESULTS

Testing bacteria ability to solubilize P and K: Purified isolates were then qualitatively examined for their ability to solubilize P and K, based on the clear zone formation around the colony (Fig. 1). The Solubility Index (SI) showed that 55 bacterial isolates were capable to solubilize P and 12 capable to solubilize K. Three bacterial isolates having highest ability to solubilize P and K were then selected for further analysis (Table 1).

Quantitative estimation of soluble P and K: The results of quantitative estimation of selected isolates to solubilize P and K showed that each isolate had different optimal time (days) to solubilize P. Isolate QC3.a.1solubilized the highest P on day 5 (50.83 mg L^{-1}), isolate QC3.a.2 on day 7 (80.61 mg L^{-1}) and isolate QC3.d.5 on day 4 (43.22 mg L^{-1}) (Fig. 2a). Similarly, K, each isolate

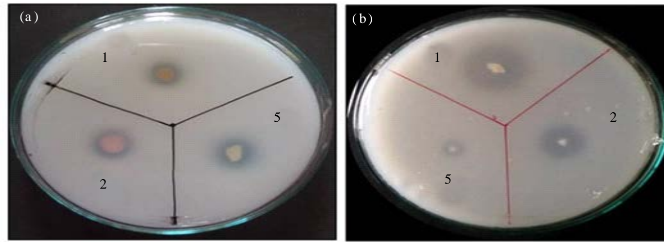

Fig. 1(a-b): Solubilization P and K of three selected isolates (a) Pikovskaya medium and (b) Aleksandrov medium

Fig. 2(a-b): (a) P solubilizing concentration and (b) K solubilizing concentration of three selected isolates. ◊: log number of cells, □: P concentration and Δ: K concentration

Table 1: Results of testing of three selected isolates in soluble P and K

Isolates	Solubility index P	Solubility index K
QC3.a.1	1.00	1.29
QC3.a.2	0.34	3.73
QC3.d.5	0.81	0.31

also had different optimal time (days). Isolate QC3.a.1 solubilize the highest K on day 25 (1.7 mg L^{-1}), isolate QC3.a.2 on day 25 (1.1 mg L^{-1}) and isolate QC3.d.5 on day 20 (1.1 mg L^{-1}) (Fig. 2b).

Bacterial morphology and physiology: The three selected isolates showed different morphological characteristic on color and elevation (Table 2). Gram staining showed that isolate QC3.a.1 and QC3.d.5 are Gram negative, which have bacilli form while the isolate QC3.a.2 is Gram negative which has cocci form (Fig. 3). Physiological identification using API 20 NE Kit showed that isolate QC3.d.5 is *Pseudomonas* sp. with similarity level of 99% and have positive result to L-arginine, urea, gelatin, D-glucose, D-mannose, D-mannitol, N acetyl glucosamine, potassium gluconate, capric acid, malic acid, trisodium citrate and phenylasetat acid tests, where as isolates QC3.a.1 and QC3.a.2 revealed to be *Burkholderia cepacia* and *Burkholderia* sp., respectively by Mubarik *et al*. (2014).

Amplification of 16S rRNA gene: The amplification result of three selected isolates based on 16S rRNA gene using 63F and 1387R primers (Marchesi *et al*., 1998) resulted an amplicon of about 1300 bp in size (Fig. 4). The phylogenetic tree showed that isolate QC3.a.1 closely related to

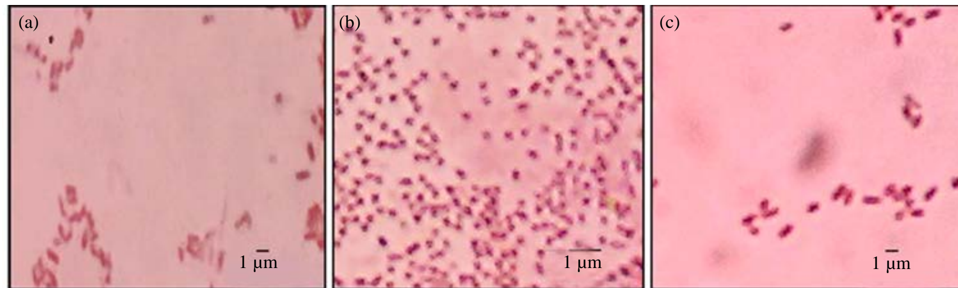

Fig. 3(a-c): Gram stain results of three selected isolates (a) QC3.a.1, (b) QC3.a.2 and (c) QC3.d.5 at 1000x magnification power

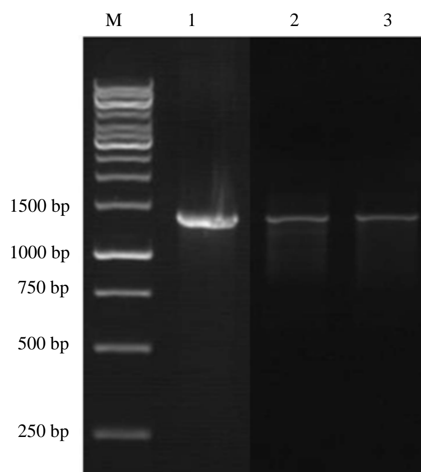

Fig. 4: Electrophoresis amplification result of three selected isolates based on 16S rRNA gene. M: Marker 1 kb, Well 1-3: Isolate QC3.d.5, QC3.a.1 and QC3.a.2, Lane 1: Isolate QC3.d.5, Lane 2: Q3.a.1 and Lane 3: QC3.a.2

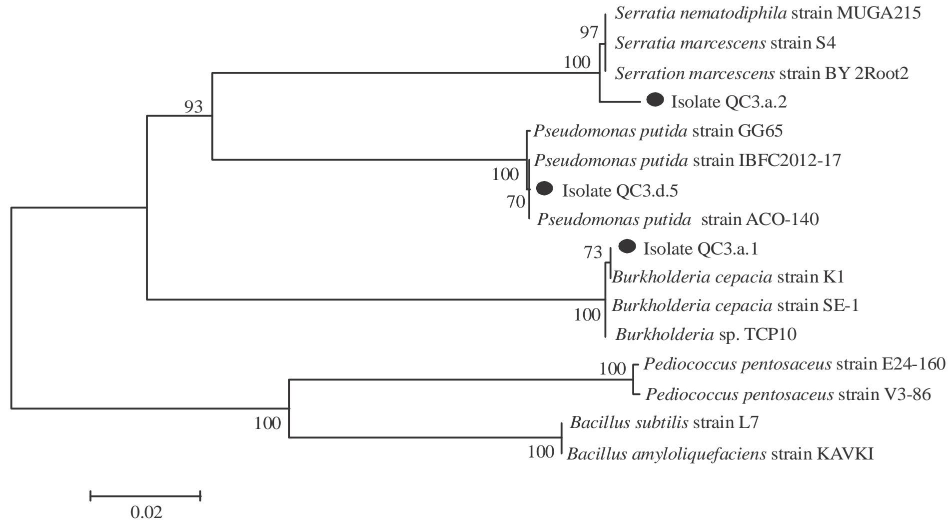

Fig. 5: Construction of phylogenetic tree of three selected isolates by 16S rRNA gene

Table 2: Characteristics of three selected isolates by morphological observation

Isolates	Characteristics morphology Shape	Edge	Elevation	Color
QC3.a.1	Round	Slip	Convex	Yellow
QC3.a.2	Round	Slip	Flat	Cream
QC3.d.5	Round	Slip	Flat	Yellow

Table 3: Result of sequences analysis 16S rRNA gene of three selected isolates at database NCBI

Isolates and description	Identity (%)	E-value	Accession No.
QC3.a.1			
Burkholderia cepacia strain K1	99	0.0	KM030037.1
Burkholderia cepacia strain SE-1	99	0.0	KF681774.1
Burkholderia sp. TCP10	99	0.0	KC833503.1
QC3.a.2			
Serratia marcescens strain By2Root2	98	0.0	KM099141.1
Serratia marcescens strain S4	98	0.0	KJ025959.1
Serratia nematodiphila strain MUGA215	98	0.0	KJ672378.1
QC3.d.5			
Pseudomonas putida strain ACO-140	100	0.0	KM349969.1
Pseudomonas putida strain IBFC2012-17	100	0.0	KC246039.1
Pseudomonas putida strain GG65	99	0.0	KJ850211.1

Burkholderia with similarity level of 99%, isolate QC3.a.2 closely related to *Serratia* with similarity level of 98% and isolate QC3.d.5 closely related to *Pseudomonas putida* with similarity level of 99-100% (Table 3 and Fig. 5).

DISCUSSION

Suliasih and Rahmat (2007) stated that bacteria grown in solid Pikovskaya medium will solubilize P, characterized by clear zone around the colony as a result of solubilizing $Ca_3(PO_4)_2$. Bashan *et al*. (2013) confirmed that the of P solubilization is due to the release of protons as H^+ where the mechanism is the same as tricalcium phosphate ($Ca_3(PO_4)_2$) solubilization, described as: $Ca_3(PO_4)_2 + 2H^+ \rightarrow 2CaHPO_4 + Ca^{2+}$.

The solubilizing index showed that two isolates potentially have the highest capability to solubilize P on Pikovskaya solid medium, i.e. QC3.a.1 and QC3.d.5, in addition to two isolates for

K on Aleksandrov solid medium, i.e. QC3.a.1 and QC3.a.2. Such variation in the solubilizing index value was due to the difference in isolates ability to secrete their extracellular organic acids. The main mechanism in solubilizing P and K is by producing organic acids (Archana *et al*., 2013; Song *et al*., 2008). In addition, the clear zone formed on Pikovskaya and Aleksandrov solid medium could not show the extent of the amount of P and K dissolved by bacteria. Lynn *et al*. (2013) confirmed that despite the narrowness wide clear zone may indicate the size of the ability of bacteria in solubilizing P and K but could not show the amount of P and K are solubilize in the medium. Therefore, dissolution testing P and K quantitatively was needed to determine how much P and K were solubilize by bacteria.

The estimation results of dissolution P and K by three selected isolates, indicated that the dissolution activity began when the number of bacteria cells began to increase. P solubilization in culture medium can be influenced by several factors, such as the composition of bacteria medium, pH changes in culture medium and the presence of phosphate solubilizing bacteria strains (Chen *et al*., 2006; Park *et al*., 2010; Aarab *et al*., 2013; Karpagam and Nagalakshmi, 2014). Solubilization of insoluble form of K, such as illite and feldspar is carried out by microorganisms due to the production of organic acids, such as oxalic acid and tartrate acid, as well as the production of polysaccharide capsular that help to solubilize minerals to release K (Sheng and He, 2006).

The results of quantitative test on bacteria to solubilize P and K showed that isolate QC3.a.1 have a capacity to solubilize 50.83 mg L^{-1} of P and 1.7 mg L^{-1} of K. Song *et al*. (2008) reported that the concentration of solubilized P was 345.9 mg L^{-1} in the culture of *Burkholderia cepacia* DA23, isolated from Gimhae soil area, Korea. Zhang and Kong (2014) reported that solubilized K concentration in bacterial culture of *Burkholderia cepacia* GL13, isolated from the rhizosphere soil around tobacco plant in Sichuan, Shandong and Hubei Province, was 0.59 mg L^{-1}. Isolate QC3.a.2 have a capability in solubilizing 80.61 mg L^{-1} of P and 1.1 mg L^{-1} of K. Perez *et al*. (2007) reported that the concentration of solubilized P ranged from 78.6 mg L^{-1}-82.7 mg L^{-1} in *Serratia marcescens,* isolated from metal surface located near Ciudad Piar of Bolivar, Venezuela. Where as, the isolate QC3.d.5 was able to solubilize 43.22 mg L^{-1} of P and 1.7 mg L^{-1} of K. Nautiyal (1999) reported that the concentration of solubilized P was 35 μg mL^{-1} in *Pseudomonas* sp., isolated from soil and plant roots from Banthra of Lucknow, India. Archana *et al*. (2013) reported that *Pseudomonas* sp., isolated from rhizosphere soil of different plants around Dharwad of Belgaum was able to solubilize approximately 8.72-20.50 μg mL^{-1} of K.

The decreased concentrations of soluble P and K was allegedly because of the reuse of soluble P and K by the culture as nutrients source for metabolic activity and the decrease in the number of bacteria cell population, which will affect the bacteria activity to solubilize P and K. Krishnaswamy *et al*. (2009) reported that phosphates are required by microorganisms to protect their cells from the environment, to synthesize nucleic acid, to build cell membranes (such as phospholipids) and to transfer chemical energy into the cells (such as ATP molecules). Gries *et al*. (2013) reported that potassium ion (K^+) play an important role in bacterial physiology, including the regulation of cytoplasmic pH, turgor pressure, the activity of intracellular enzymes and trans-membrane potential.

The result of 16S rRNA gene amplification showed that isolate QC3.a.1 was closely related to *Burkholderia*, isolate QC3.a.2 was closely related to *Serratia* and isolate QC3.d.5 was closely related to *Pseudomonas putida*. *Burkholderia*, *Serratia* and *Pseudomonas* are member of bacteria group having capability in solubilizing P and K by producing organic acids (Rodriguez and Fraga,

1999; Archana *et al*., 2013) and promoting plant growth through the production of IAA (Patten and Glick, 2002). *Burkholderia* is known as N-fixing bacteria and exhibited antifungal activity (Stephen and Jisha, 2011; Mubarik *et al*., 2014). *Serratia* is potential to inhibit growth and aflatoxin production of *Aspergillus parasiticus* (Wang *et al*., 2013). *Pseudomonas* have an ability in protecting plants from pathogens and potentially by useful as biocontrol agent applicable in green house and field (Arshad and Frankenberger Jr., 1993). *Burkholderia*, *Serratia* and *Pseudomonas* are efficient to be use as biological fertilizer on agricultural land (Rodriguez and Fraga, 1999; Ghaderi *et al*., 2008; Lavania and Nautiyal, 2013).

CONCLUSION

Three selected isolates have a highest capacity in solubilizing P and K on Pikovskaya and Aleksandrov solid medium. Isolate QC3.a.2 has the highest capability in solubilizing P on Pikovskaya liquid medium while isolate QC3.a.1 is the best solubilizer of K on Aleksandrov liquid medium. Three selected isolates are Gram-negative bacteria. The isolate QC3.a.1 is closely related to *Burkholderia*, QC3.a.2 is closely related to *Serratia* and QC3.d.5 is closely related to *Pseudomonas putida*.

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